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THE HEAT-STABLE ENTEROTOXIN(S) OF *YERSINIA ENTEROCOLITICA* IN FOODS

FINAL REPORT

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The heat-stable enterotoxin of Yersinia enterocolitica (YST) was partially purified by $(NH_4)_2SO_4$ precipitation, XAD-2 chromatography, and gel permeation chromatography. Most strains of Y. enterocolitica can produce YST. Production was optimal at 26°C, occurred at refrigerator temperatures, and was not observed at 37°C. YST was produced throughout the pH range of 6.0-8.5.

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Yersinia enterocolitica is a recently recognized food-borne pathogen, which causes severe abdominal cramping and diarrhea. The pathogenic mechanism of Y. enterocolitica appears to be infection and invasion of the ileum, upper colon and surrounding lymphatic tissue. Y. enterocolitica can grow at refrigerator temperatures; it also produces a heat-stable enterotoxin (YST) similar to Escherichia coli heat-stable enterotoxin (EST). Although YST is probably not involved in the infectious disease because it is not produced at body temperature (37°C), YST may be involved in as yet unreported cases of food-borne intoxication. The purpose of this study was to determine the conditions of YST production in media and foods, to purify and characterize YST and to study the toxicology of YST in tissue culture and whole animals.

Using the infant mouse assay, we have determined that 40 of 55 strains of Y. enterocolitica and Y. enterocolitica-like bacteria produce YST within 48 hours at 26°C (Appendix A). A complex medium consisting of 2% Casamino Acids, 1% yeast extract, 0.4% glucose pH 8.5 (CYG) was found to be optimal for production of YST (Appendix A). YST is produced at low levels at 37°C by two strains of Yersinia kristensenii (sucrose nonfermenting Y. enterocolitica) within 72 hours. 10 of 12 strains of Y. enterocolitica and Y. kristensenii produced YST within 72 hours at 26°C and 20°C and within 96 hours at 15°C. At 7°C seven of twelve strains tested produced YST within 7 days (Appendix B). There was no apparent difference in YST production at pH 6.0 to pH 8.5 in CYG at 26°C (Appendix C). Evaluation of YST production in foods has been unsuccessful (Appendix D).

Preliminary purification work has been more successful. YST is a low molecular weight polypeptide, methanol-soluble and stable to heat at 100°C for 10 minutes. Our purification scheme is as follows:

1. Precipitation of YST at 90% saturated $(NH_4)_2SO_4$.

2. Adsorption chromatography with Amberlite XAD-2, the toxin is eluted with 1% acetic acid, 99% methanol.

3. Gel permeation chromatography with Sephadex G-50 superfine, elute with PBS (pH 7.4).

Disc electrophoresis of ammonium sulfate precipitate and the toxin fraction from Amberlite XAD-2 was done using 15% acrylamide small-pore gels. The gels were stained with Coomasie blue G-250. The ammonium sulfate precipitate gel showed 3 major and many minor bands, there were 2 major and 3 minor bands visible in the XAD-2 toxic fraction. One of the major bands on both gels corresponded to YST (determined by elution of unfixed gels with PBS, pH 7.4), this band migrated very close to the dye front. The K_{av} of YST on Sephadex G-50 2.5 x 43 cm was determined to be circa 0.4. Attempts are being made to improve the resolution on G-50 by doubling the column length. After gel permeation chromatography, we anticipate further purifying YST using FPLC or MonoQ HR 5/5 column, which is a strong anion exchange column.

APPENDIX A

This project was initiated by acquiring 55 cultures of Yersinia enterocolitica from other investigators and screening these strains for their ability to produce the heat-stable enterotoxin (YST). The goal was to identify high level YST producers. Such high level YST-producing strains would be useful in the later purification of YST.

Upon arrival, each Y. enterocolitica culture was transferred to a TSA (trypticase soy agar) slant. For assessing their abilities to produce YST, each strain was inoculated from the TSA slant into a 250 ml Erlenmeyer flask containing 25 ml of a casamino acids-yeast extract-glucose medium (2% casamino acids, 1% yeast extract, and 0.4% glucose, pH 8.5). This medium was previously used by Pai and Mors (1) in their studies of YST production. The cultures were incubated in a shaking incubator for 48 h at 26°C. The cultures were then centrifuged at 12,100 xg for 10 min. The supernatant fluid was decanted and filter sterilized through a 0.45 µm Millipore filter. The culture fluids were then frozen until testing for YST. Evidence for YST was obtained with the infant mouse bioassay essentially as described by Dean et al. (2). CF-1 Harlan/Sprague-Dawley mice of 4 days of age were used. Sterile culture fluid supernatants with 1 drop of 2% Evan's blue per ml were injected directly into the milk-filled stomachs of these mice. 100 µl of each sample was injected into each of 3 mice. The mice were held at room temperature for 4 h. The mice were then killed by cervical dislocation. The entire intestinal tract was removed and weighed. The remaining carcass was also weighed. The ratio of gut weight to remaining body weight was calculated. A ratio of 0.033 or greater is considered positive; control ratios ranged from 0.050 to 0.065.

Of the 55 strains tested, 40 (72.7%) were found to be YST producers. A summary of the results is shown in Table 1. The ratios for the YST producers ranged from 0.095 to 0.150. YST producers were found in the 0:3, 0:5, 0:6,30, 0:7,8, 0:8, 0:16, and 0:28 serotypes. Most of the biochemically atypical strains failed to produce YST.

The ratio of gut weight to remaining body weight does not allow identification of the most productive YST-producing strain. The ratio is not correlated particularly well with the amount of YST. The best method to obtain information about the relative amounts of YST is to use serial dilution of the culture supernatant and record the lowest dilution that elicits a positive ratio.

Thirteen of the YST-producing strains were subjected to this titration procedure. Table 2 shows the results of these titrations by giving the original ratio and the maximal dilution that still provided a positive ratio. While the ratios for these 13 strains ranged from 0.095 to 0.150, the maximal dilutions that elicited positive responses ranged from 1/1 to 1/128. Ten of the 13 strains exhibited toxin titers of 1 to 10. Strain 106, an 0:5 strain, had a toxin titer of 32 while strain G14A, a 0:28 strain, exhibited a toxin titer of 128. In all cases, the dilutions were made in phosphate-buffered saline.

From this data, it would seem to be prudent to choose strain G14A for our YST purification efforts. However, we are not convinced that a O:28 strain would be a wise choice since their environmental distribution is limited apparently to shrews, rodents, and water (3). Also, the O:28 strains are capable of YST production at 37°C, a trait not shared by other serotypes of Y. enterocolitica (3). Consequently, we have not yet made our final choice of the strain to be employed in the YST purification studies.

For the studies on the optimal medium for YST production, ten strains of Yersinia enterocolitica and two strains of Y. kristensenii were employed.

Ten strains of Yersinia enterocolitica and two strains of Yersinia kristensenii (sucrose non-fermenter) were used in this study. Sterile culture fluid (0.1 ml/mouse into the stomach) was assayed for toxin by the infant mouse assay using three, 3-4 day old mice for each test. A ratio of gut weight/carcass weight greater than 0.083 was considered positive (Pai & Mors, 1978).

Six media were studied for enterotoxin production at 26°C. Three were defined media: 1) Minimal medium + 1% glucose (Vera & Power, 1980), 2) Minimal medium for optimal production of E. coli heat-stable toxin (MEC, Staples, et al., 1980), 3) MEC + Vitamin mix. Three were complex media: 1) MEC + 0.6% yeast extract (MEY), 2) 2% Casamino acids, 1% yeast extract, 0.4% glucose, pH 8.5 (CYG, Pai & Mors, 1978), 3) Medium for optimal growth of Y. enterocolitica (OGM, Schiemann, 1980). The pH of all the defined media were 7.7; none of the strains of Y. enterocolitica produced toxin in defined media. The pH of OGM was 7.7; only three out of six positive strains produced toxin in this medium. Toxin was produced by all positive strains in both MEY (pH 7.6) and CYG (pH 8.5). The most toxin was produced in CYG.

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TABLE 1

YST Production by Selected *Yersinia enterocolitica* Strains

Serotype	Biotype	Strain No.	Original Source	YST Production
0:3	4	C108-76	human feces	+
0:3	4	C122-76	human abscess	+
0:3	4	700	human	+
0:3	-	IP134	unknown	+
0:3	4	6806	unknown	+
0:3	4	6809	unknown	+
0:3	4	6810	unknown	-
0:4,32	2 (atypical)	M46-76	raw ground beef	-
0:4,32	2 (atypical)	M49-76	smoked sausage	-
0:4,32	1	M594-79	water	-
0:4,32	1 (Rham.+)	M170-80	water	-
0:5	3	106	raw fish	+
0:5	3	84	pig tonsils	-
0:5	-	IP124	unknown	+
0:5(5A)	1	14011	human stool	+
0:5(5A)	1	15750	human stool	+
0:6,30	1	M52-75	milk	-
0:6,30	1	M63-76	water	+
0:6,30	1	M403-78	ground beef	+
0:6,30	-	IP102	unknown	+
0:6,31	1	102	raw milk	-
0:7,8	1	29828	human stool	+
0:8	-	WA	human	-
0:8	-	WA eye +	human	+
0:8	-	WA ETBR37B	from WA	+
0:8	-	A2635	chocolate milk	+
0:8	-	Y7P	human	+
0:8	-	Y7N	from Y7P	+
0:8	-	TAMU Ag -	unknown	+
0:9	-	IP383	unknown	-
0:16	-	IP475	unknown	+
0:28	-	11051A	shrew	+
0:28	-	9021B	shrew	+
0:28	-	9019A	shrew	+
0:28	-	1001	shrew	+
0:28	-	1003B	shrew	+
0:28	-	G14A	water	+
0:28	-	G13A	water	+
0:28	-	G2	water	+
0:28	-	9036	small rodent	+
0:28	-	9008	small rodent	+
0:28	-	1030	small rodent	+
0:28	-	IP1474	water	+
0:28	-	10016B	shrew	+
unk.	1	11-R-A	oysters	+
unk.	2	45-CM-A	oysters	+
unk.	1 (Rham.+)	5-28-R-A	shrimp	-
unk.	1 (Rham.+)	202	shrimp	-
unk.	1 (Rham.+)	C-35-R-A	crab	-
unk.	-	L2	food	+
unk.	-	L11	food	-
unk.	-	L28	food	+
unk.	-	5L32	food	+
unk.	-	1970-71	food	-
unk.	-	1474	food	+

TABLE 2

Toxin Titer Produced by Selected Strains of *Yersinia enterocolitica*

Strain No.	Serotype	Ratio	Toxin Titer
C122-76	0:3	0.095	1
6809	0:3	0.132	8
106	0:5	0.110	32
M403-78	0:6,30	0.124	8
29828	0:7,8	0.134	8
A2635	0:8	0.126	4
WA eye +	0:8	0.129	2
IP475	0:16	0.145	10
G14A	0:28	0.109	128
G2	0:28	0.143	10
IP1474	0:28	0.150	4
11-R-A	unk.	0.138	10
45-CM-A	unk.	0.115	10

APPENDIX B

The studies of the effect of temperature on enterotoxin (YST) production by Yersinia enterocolitica and Y. kristensenii in casamino acids-yeast extract-glucose (CAY) medium, pH 8.5, have been completed. Twelve strains were chosen: 2 Y. kristensenii strains (one each of serotype 0:16 and 0:28) and 10 Y. enterocolitica strains (3 of serotype 0:8, 2 each of serotypes 0:3 and 0:6,30, and 1 each of serotypes 0:4,32, 0:5, and 0:9). Incubations were done at 5 temperatures: 37°C, 26°C, 20°C, 15°C, and 7°C. Samples were taken periodically for YST analysis in the infant mouse assay. The data from the temperature study are summarized in T ble 1. The rate and extent of YST production was greatest at 20°C and 26°C. Only the Y. kristensenii strains produced YST at 37°C. At 15°C, YST production was delayed but still occurred with 8 of the 10 Y. enterocolitica strains by the end of four days of incubation. At 7°C, YST production was delayed even further with only 5 of 12 strains of Y. enterocolitica producing YST after 7 days of incubation. There is no correlation between YST production at any temperature and serotype. The Y. kristensenii strains were both able to produce YST within 7 days at 7°C.

Table 1: Temperature Study

Temp.	Strain	Toxin titer/0.1 ml 6 hour	1 day	2 day	3 day	4 day
37°C	C108-76	--[1]	--	--	--	
	M49-76	ND[2]	ND	--	--	
	M52-75	ND	ND	--	--	
	M403-78	ND	ND	--	--	
	106	ND	ND	--	--	
	1P383	ND	ND	--	--	
	1P475[3]	--	--	--	1	
	CDC A2635	ND	ND	--	--	
	WA	ND	ND	--	--	
	6809	ND	ND	--	--	
	G14A[3]	--	--	(1)	2	
	FRI YE13	ND	ND	--	--	
		6 hour	1 day	2 day	3 day	4 day
26°C	C108-76	ND	--	1	8	
	M49-76	"	--	--	(1)	
	M52-75	"	--	--	--	
	M403-78	"	--	4	16	
	106	"	--	32	64	
	1P383	"	--	5	16	
	1P475[3]	"	--	2	8	
	CDC A2635	"	--	1	16	
	WA	"	--	4	8	
	6809	"	--	4	8	
	G14A[3]	"	--	64	64	
	FRI YE13	"	--	8	64	

		6 hour	1 day	2 day	3 day	4 day
20°C	C108-76		ND	--	1	4
	M49-76		"	--	--	--
	M52-75		"	--	(1)	--
	M403-78		--	1	8	4
	106		--	4	16	16
	1P383[3]		--	2	8	8
	1P475[3]		ND	--	4	8
	CDC A2635		--	4	4	8
	WA		--	2	4	2
	6809[3]		--	2	2	4
	G14A[3]		--	32	128	64
	FRI YE13		--	(1)	32	32

		2 day	3 day	4 day
15°C	C108-76	ND	--	1
	M49-76	ND	--	--
	M52-75	ND	--	--
	M403-78	--	1	4
	106	--	2	8
	1P383[3]	--	1	4
	1P475[3]	--	1	8
	CDC A2635	--	1	4
	WA	ND	--	4
	6809[3]	ND	--	2
	G14A[3]	--	2	8
	FRI YE13	--	2	4

		5 day	6 day	7 day
7°C	C108-76	--	--	--
	M49-76	--	--	--
	M52-75	--	--	--
	M403-78	--	--	2
	106	--	--	4
	1P383[3]	--	--	--
	1P475[3]	--	2	8
	CDC A2635	--	--	2
	WA	--	--	--
	6809[3]	--	--	1
	G14A[3]	--	4	4
	FRI YE13	--	1	4

[1] Toxin test done, no toxin present.
[2] ND = not done, toxin test not done.
[3] These strains are Y. kristenseniі; the others are Y. enterocolitica.

APPENDIX C

The effect of pH (in culture media) on the enterotoxin (YST) production by Yersinia enterocolitica and Y. kristensenii was studied. Four strains were used: 3 Y. enterocolitica (1 serotype O:6,30; 1 serotype O:5; 1 serotype O:8) and 1 Y. kristensenii (serotype O:28). The medium - 2% casamino acid, 1% yeast extract, 0.4% glucose (CYG) - was adjusted to the desired pH before autoclaving. Six pH levels were used: 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The reason for choosing those pH values is that most foods (especially the foods where Y. enterocolitica has been found) fall into this range. The cultures were incubated with shaking for 48 hours at 25°C. There was no difference in growth for any of the strains at any pH. The toxin titers at 48 hours are shown in Table I.

Studies on YST production in foods have been started. The foods chosen are pork slurry, skim milk, and tofu slurry. The tofu is being used because of the recent outbreak of yersiniosis from tofu in Washington State. Purification work has also been started. Mainly we have done a quick experiment to see if we can remove any interfering low molecular weight compounds by desalting through Sephadex G-25 with PBS (pH 7.4) as an eluent.

Table I. Effect of pH on YST production (titer/0.1 ml)

		pH					
Strain #	Serotype	6.0	6.5	7.0	7.5	8.0	8.5
14	O:6,30	-	-	-	-	1	-
19	O:5	8	32	32	16	16	8
34	O:8	1	2	1	2	2	1
53	O:28	16	16	16	8	16	8

APPENDIX D

One experiment on growth of Yersinia sp. and toxin (YST) production in foods was done. Three foods were used: raw pork (75 g), tofu (75 g + 75 ml sterile tap water) and reconstituted skim milk (25 ml). None of the foods was sterile; therefore, we used one control - inoculated with 1 ml of sterile PBS - for each food. We also inoculated separate samples of each food with 3 strains of Yersinia: 14, 34 and 53 (see earlier reports for details on these strains). The foods were incubated for one week at 27°C; samples for total plate count (on TSA) and Y. enterocolitica isolation (CIN agar) (1) were taken every day. Samples for toxin testing were taken at days 6 and 7. None of the Yersinia strains grew in either the tofu or the pork. All of the Yersinia strains grew in the milk. Because the Yersinia strains did not grow in pork or tofu, toxin testing was not done.

END